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13. ABSTRACT *(Maximum 200 words)*

Cytoskeleton remodeling is crucial in many cellular events, including cell adhesion, spreading and motility. The Rho family of small GTPases (Rho, Rac and Cdc42) are signal transducers that regulate cytoskeleton dynamics. However, little is known about the mechanisms by which Rho GTPases induce cytoskeletal changes. Here we show that Rac1 is important for cell spreading, a biological process in which the cytoskeleton is highly active. Overexpression of dominant negative or constitutively active Rac1 significantly impairs the ability of the cells to spread. Rac activity is high only during early stages of spreading. The activity of p-21activating kinase (PAK), an effector molecule for Rac and Cdc42, is also high only during the early stages of spreading. Overexpression of catalytically active PAK inhibits cell spreading and decreases myosin phosphorylation. Myosin is the cytoskeleton protein which provides the force generating ability to the actin cytoskeleton. Treatment of cells with myosin inhibitors also inhibits cell spreading. In vitro and in vivo studies revealed a novel target for PAK, myosin light chain kinase (MLCK), the enzyme that phosphorylates the light chain of myosin II. Activated PAK phosphorylates MLCK and inhibits its ability to phosphorylate MLC. Thus, PAK appears to modulate cytoskeleton changes by phosphorylation and downregulation of MLCK.

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Lurayone Sanders 8-31-98

PI - Signature Date

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Introduction

Tumor invasion and metastasis are the major cause of cancer mortality. This is particularly true for breast cancer since metastasis of the primary tumor leads to tumor inaccessibility and, consequently, greater mortality. Many studies have shown the importance of signaling molecules in changes that are associated with transformed phenotypes (1-3). In fact, many signaling molecules are affiliated with cytoskeleton changes that accompany the motile or metastatic phenotype. The Rho family of small GTPases (Rho, Rac and Cdc42) are members of the Ras superfamily; all of which regulate cell function via conversion between a GTP-bound active state and a GDP-bound inactive form. Recently, it has become clear that the Rho family mediates morphological and cytoskeletal changes of both normal and transformed cells (4,5). Rho activation leads to stress fiber formation and focal adhesions. Activation of Rac leads to membrane ruffles and lamellipodia formation. Similarly, Cdc42 regulates the extension of actin filament bundles into filopodia. The mechanisms by which the Rho family of GTPases regulate cytoskeleton remodeling is not well understood.

Dynamic rearrangement of the cytoskeleton is, in part, driven by actin polymerization and actin-myosin interactions. Myosins are mechanoenzymes which generate force along actin filaments and, thus, are crucial for cell movements, including cytokinesis, pseudopod formation, polarized growth and cell migration (6-8). Changes in the expression of myosin isoforms have been linked to the transformed phenotype in both melanoma and breast cancers (9-11). Recently, Rho has been shown to regulate myosin activity though Rho kinase, an effector molecule for Rho (12,13). Rho kinase phosphorylates myosin phosphatase and inhibits its function, concequently acting to increase myosin phosphorylation. Therefore, Rho GTPase seems, in part, to regulate cytoskeleton changes by activating Rho kinase and thereby increasing myosin phosphorylation and effecting its force generating abilities.

In this grant we proposed to examine the effects p-21 activating kinase (PAK), an effector molecule for Rac and Cdc42, had on myosin phosphorylation during migration of breast cancer cells (Aims 2 & 3). Our results in this report indicate that PAK acts to inhibit myosin phosphorylation, the opposite effect that Rho kinase had on myosin. This inhibition is not due to a direct interaction of PAK with myosin. Our data suggest that this effect is due to PAK phosphorylation and inhibition of myosin light chain kinase (MLCK), a kinase known to phosphorylate myosin and thereby regulate its activity.

Materials and Methods

Transfection of cells with Semliki Forest Virus

The cDNA encoding PAK1 WT and mutation T423E were all expressed in cells using the Semliki Forest Virus (SFV) Gene Expression System (Life Technologies, Gaitherburg, MD). The cDNAs were PCR amplified using primers that contained a BamH1 restriction enzyme site and a myc tag at the 5' end. These constructs were subcloned into the BamH1 site of the Semliki Forest vector pSFV3. In vitro transcription of linearized pSFV3 constructs and pSFV-Helper2 was performed using SP6 RNA polymerase. RNA transfection of BHK-21 cells was done by electroporation as previously described (14), yielding recombinant viral stocks of approximately 10^7 plaque-forming units (pfu)/ml. Viral stocks were stored at -80°C until used. Virus was activated per manufactures instruction, after which BHK-21, MDA 435, HS 578T and ZR- 75 cells were infected in serum-free media. Transfection efficiency of recombinant virus was routinely greater than 95% in most cell lines examine. Cells were allowed to express protein for 6 to 8 hour after infection in serum free media before use in experiments.

Cell Adhesion Assay

Cell adhesion assays were performed as previously described (15). In brief, cells were suspended in basal media (GMEM, Life Technologies) containing no serum and seeded in 6-well plastic microtiter plates containing coverslips pre-coated with 20ug/ml

fibronectin (Sigma). Cells were allowed to adhere and at various time points were fixed in 4% paraformaldehyde. For inhibition studies, various concentrations of BDM (2,3-Butanedione Monoxime, Sigma) and ML-7 (Calbiochem) were placed in media prior to cells.

Immunofluorescence

Cells attached to coverslips were fixed in 4% paraformaldehyde for 20 min, then permeabilized with 0.5% Triton X-100 for 20 min. Coverslips were incubated with anti-myc at 1:300 (9E10) or anti-β Gal at 1:5,000 (Promega, Madison, WI) for 1 hr, and washed with PBS. Cells were incubated for 1 hr with rhodamine phalloidin 1:500 (Sigma Chemical Co.) and FITC-conjugated anti-mouse IgG 1:300 (Cappel Laboratories, Cochranville, PA), washed with PBS and mounted in Fluoromount-G (Southern Biotechnology Associates, Biringham, AL). Slides were visualized using a Nikon Labphot-2 DFX-DX epifluorescence microscope. Images were photographed with a Nikon FX-35 DX 35-mm camera back and Kodak print film (Tmax).

Immunoblots

30 μl of cell lysate from control (nontransfected) or transfected BHK-21 cell (35 mM dish) were run on 15% SDS-PAGE gels, transferred to PVDF membrane using semi-dry transfer apparatus, stained with a 2% Ponseau S solution to check transfer then rinsed in water and blocked with buffer containing 10% goat serum, 3% BSA in 10mM Hepes. Blots were incubated with anti-Ser19 MLC-P, pp2b, for 1 hr (16). Protein bands were visualized with horseradish-peroxidase-conjugated anti-rabbit IgG (Pierce) and chemiluminescence (Pierce).

In-Gel Kinase Assay

PAK kinase activity was detected in gel by its ability to phosphorylate a $p47^{phox}$ peptide corresponding to amino acids 297-331 as previously described (17).

Rac Activity Assay

Cells were placed in microtiter plates coated with fibronectin and at various times lysed with cold RIPA buffer (containing PMSF, leupeptin, aprotinin and orthovanadate) in the presence of 10μg of GST-PBD (the domain of PAK which binds the GTP forms of Rac and Cdc42). Clarified cell lysates were incubated 1hr at 4°C with Glutathione Sepharose 4B beads in binding buffer (25mM Tris-HCL, 1mM dithiotreitol, 30mM $MgCl_2$, 40 mM NaCl, 0,5% NP40). Beads were pelleted and washed 5 times in binding buffer then resuspended in 20μl of sample buffer. Proteins were separated on a 12% SDS-PAGE gel, transferred to nitrocellulose membrane and blotted using anti-Rac.

RESULTS

Detailed cellular analysis of breast cancer cell lines has been hindered by the low protein expression and number of transfected cells that microinjection and other traditional methods render. Therefore, we perfected a viral gene expression system to overcome this problem. This system has allowed us to transiently express Rho family GTPases and PAKs with an efficiency of greater than 95% in many breast cancer cell lines (figure 1 left panel, table 1). Consequently, we are now able to study a population of cells which can be placed into bioassays such as migration or adhesion assays.

During cell spreading the cytoskeleton is in a dynamic state of remodeling (18,19). The spreading edge of a cell is often compared to the leading edge of a migrating cell and collectively these are referred to as moving edges. Furthermore, cell spreading is a prerequisite for cell migration. To understand the mechanism by which the Rho family of small GTPases exerts its effect on the cytoskeleton, we transfected cells with Rac1 and its mutant forms, and performed cell adhesion assays. BHK-21 cells were transiently transfected using the viral gene expression system and allowed to overexpress Rac1 WT, dominant negative Rac1 (T17N), and constitutively active Rac1 (Q61L) for 6 hr. After expression cells where placed on fibronectin coated coverslips and allowed to adhere and spread for 2 hr. Cells expressing Lac Z or Rac1 WT attached and spread normally (figure

1). However, cells expressing dominant negative Rac1 (T17N) strongly inhibited spreading (figure 1). BHK-21 cells expressing constitutively active Rac1 (Q61L) started to spread but spreading was inhibited at an intermediate stage, characterized by peripheral filamentous actin (figure 1). To confirm that the effects we observed with Rac1 (T17N) were truly due to an inhibition of spreading, and not due to cell death or the inhibition of cells attachment, we placed cells expressing Rac1 WT, Q61L, T17N and Lac Z in 24-well microtiter plates coated with fibronectin and performed adhesion assays at various time points (figure 2). At every time point control (no virus) cells and transfected cells showed no difference in their ability to adhere. Thus, the effects seen with Rac1 (T17N) is genuinely due to an inhibition of cell spreading. Overexpression of both constitutively active or dominant negative Rac seem to interfere with the ability of the cell to spread. This observation suggests that it is the cycling of Rac between a GDP and GTP bound form thst is important for normal cell spreading to occur.

To further understand Rac's we looked at the activity of Rac during adhesion and spreading. Cells were transfected with Rac1 WT and Q61L, allowed to express protein for 6hr, then placed in an adhesion assay and lysed at various time points. Cell lysates were incubated with a domain of PAK which specifically binds GTP- bound Rac or Cdc42. This assay allows us to detect Rac1 in its activated state. Activation of Rac1 WT was detected at 15 min and reduced to base line activity by 120 min, the time by which most of the cells have completed spreading (figure 3). This data indicates that adhesion alone is sufficient to activate Rac1. Together these results suggest that the early stages of cell spreading are dependent on Rac1 activity.

To investigate the mechanism by which Rac1 exerts its effect on cell spreading we examined the effects of PAK 1, a kinase which is activated by Rac and Cdc42, on cell adhesion and spreading. Control cells or cells overexpressing Lac Z, PAK1 WT, or mutated catalytically active PAK1 (T423E) were placed in microtiter plates and allowed to spread. At various times cells were lysed and in-gel kinase assays were performed.

Endogenous Pak activity was highest during the first 15 min of cell spreading, and gradually decreased to nearly base line activity by 120 min, or when cells were fully spread (figure 4). Similar experiments were performed on fibronectin coated coverslips and observed using immunofluorescence. After 2 hr control and PAK1 WT cells attached and spread normally. Phalloidin stain reveals a typical fibroblast-like morphology, with numerous stress fibers (appendix, figure 1A). In cells expressing PAK1 T423E attachment to fibronectin was normal, but spreading was dramatically inhibited as evident by the round cell morphology (appendix, figure1A).

Cell spreading has been suggested to be a actin-myosin mediated event (18,19). To confirm myosins role in spreading of BHK-21 cells we used inhibitors of myosin and myosin light chain kinase (MLCK). BDM has been reported to be a reversible inhibitor of myosin ATPase activity and has been shown to inhibit postmitotic cell spreading (Mit.). At a concentration of 20mM BDM inhibited BHK-21 cell spreading even at the 90 min time point in an adhesion assay (appendix, figure 1B). This inhibition was reversible: when BDM was washed out after 45 min, spreading resumed and occurred normally (appendix, figure 1B). This inhibition of cell spreading was dose dependent when tested at concentrations from 2 to 50 mM (data not shown). Similar results were obtain with the MLCK inhibitor ML-7 (data not shown) (20).

An increase in phosphorylation at Ser-19 in MLC is essential for force generation by myosin II. During postmitotic cell spreading, phosphorylation of this site is high when compared to completely spread cells (21). To further investigate the mechanism by which PAK1 exerts its effect on the spreading of BHK-21 cells we analyzed the effect PAK1 had on MLC phosphorylation. BHK-21 cells transfected with PAK1 T423E, or control cells (non-transfected) were allowed to attach and spread on a fibronectin matrix, then lysed at various time points. Immunoblot analysis was performed using an antibody which recognizes the Ser-19 phosphorylated form of MLC (16). During cell spreading the control cells show a gradual increase in MLC phosphorylation, with the maximum at

the 45 min time point (appendix, figure 2). However, PAK1 T423E cells show little to no phosphorylation of MLC at any of the time points, consistent with the inability of these cells to spread (appendix, figure 2). Cells transfected with Lac Z or Pak WT yielded similar result as control cells (data not shown). These data suggest that in vivo catalytically active PAK1 acts to inhibit phosphorylation of MLC on Ser-19.

The calcium-calmodulin dependent myosin light chain kinase (MLCK) phosphorylates MLC on Ser-19 and is known in vivo to be responsible for promoting the force generating ability of myosin II. Therefore, in order to understand PAK's role in decreasing phosphorylation of MLC, we looked at its effect on MLCK. In vitro phosphorylation experiments demonstrate that PAK 1 can phosphorylate MLCK and this phosphorylation is independent of calmodulin (appendix, figure 3). Interestingly, when MLCK is phosphorylated by PAK its activity towards MLC is decreased by ~50 % (appendix, figure 3). This data suggest that catallytically active PAK inhibits MLC phosphorylation by phosphorylating MLCK and downregulating its activity.

To test the ability of PAK to inactivate MLCK in vivo cells were transfected with PAK1 WT and T423E. Cells were lysed and MLCK was immunopercipitated and assayed for activity. MLCK immunopercipitated from PAK T423E cells showed a significant decrease in activity when compared to MLCK from control or PAK WT expressing cells (appendix, figure 4). This data confirms our in vitro data that PAK phosphorylation of MLCK inhibits its activity.

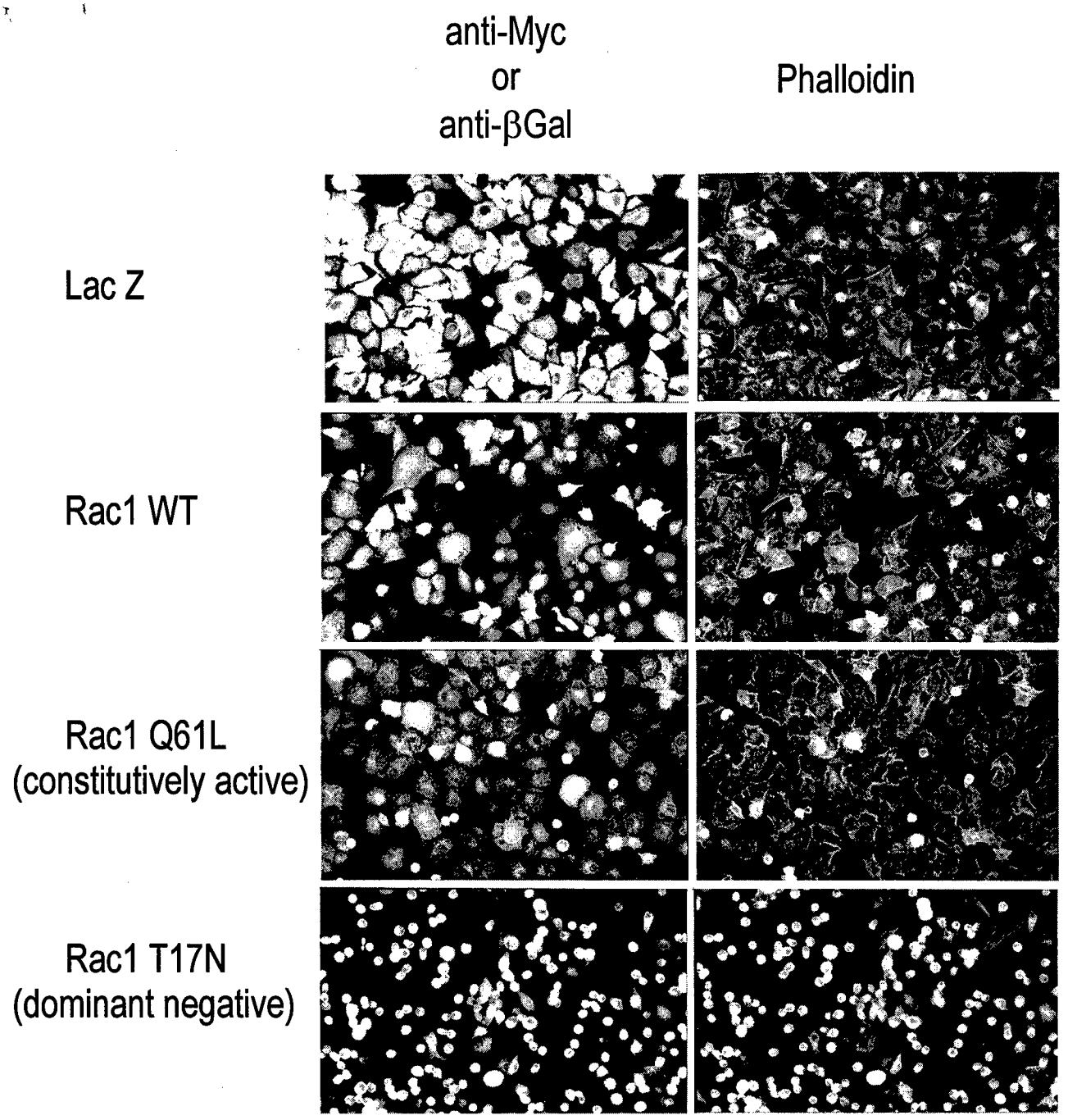

Figure 1: Adhesion and spreading of BHK-21 cells transfected with Rac 1 mutants. BHK-21 cells were infected with virus containing Lac Z (control), Rac WT, Q61L (constitutively active), T17N (dominant negative) cDNAs, and were allowed to express protein for 6 hr. Cells were harvested, plated on cover slips coated with fibronectin, and allowed to adhere and spread for 2 hrs. After 2 hrs of adhesion Rac 1 WT and Lac Z had a normal fibroblast-like morphology with numerous stress fibers. The Rac 1 T17N cells still attached to the fibronectin matrix, but spreading was strongly inhibited. Rac 1 Q61L cells had a rounded morphology with peripheral filamentous actin staining.

Table 1: Transfection efficiency of different cell lines with Semliki Forest Virus

Cell line	Cell Type	% Transfection
BHK-21	fibroblast	>95%
MDA 435	breast cancer	>95%
Hs 578T	breast cancer	>95%
ZR 75	breast cancer	>95%
T47D	breast cancer	~40%

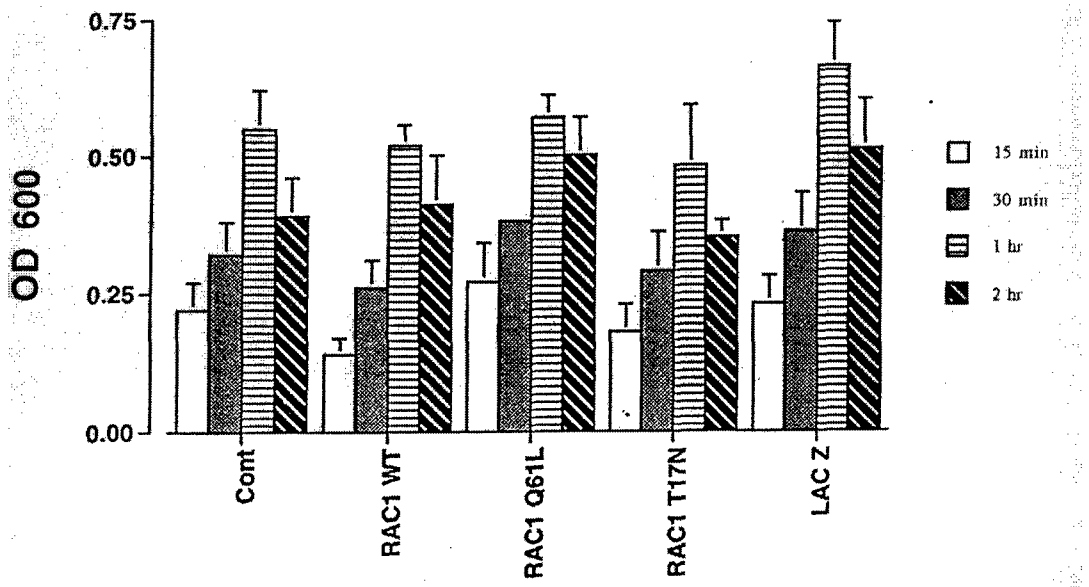

Figure 2: Adhesion assay of BHK-21 cells transfected with Rac1 and mutants. Cells were infected with Lac Z (as a control) or Rac1 and mutations, allowed to express for 6 hr, harvested, and placed in 24-well plates coated with fibronectin. At various time points wells were washed 3 times, fixed and stained with crystal violet. Stain was dissolved and read at OD 600. At all time points, control (no virus), Lac Z, Rac1 WT, Q61L, T17N cells showed no difference in their ability to adhere. This demonstrates that all the cells were equally capable of attaching to a fibronectin matrix. Consequently, the data in figure 1 with Rac1 T17N is truly due to the cells inability to spread.

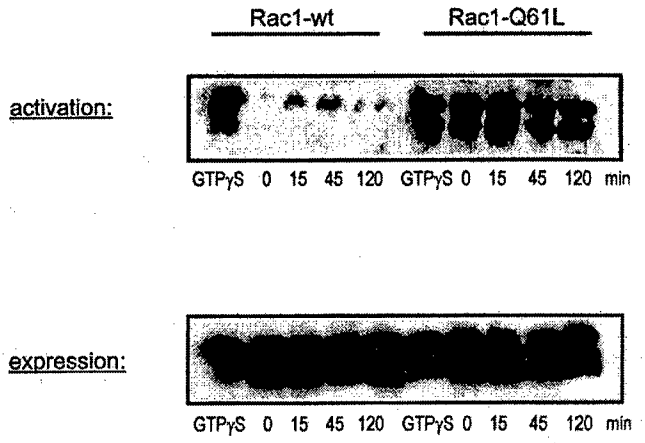

Figure 3: Activation of Rac1 by adhesion. Cells were infected with Rac1 WT and Q61L virus, allowed to express protein for 6 hr, then placed in an adhesion assay and lysed at various time points. Cell lysates were incubated with a domain of Pak which specifically binds Rac-GTP. This assay allows us to detect Rac in its activated state. Constitutively active Rac1 Q61L is detectable in all lanes including time 0 (positive control). Activation of Rac1 WT was detectable as early as 15 min and decreased by 120 min. This indicates that adhesion alone is sufficient to activate Rac.

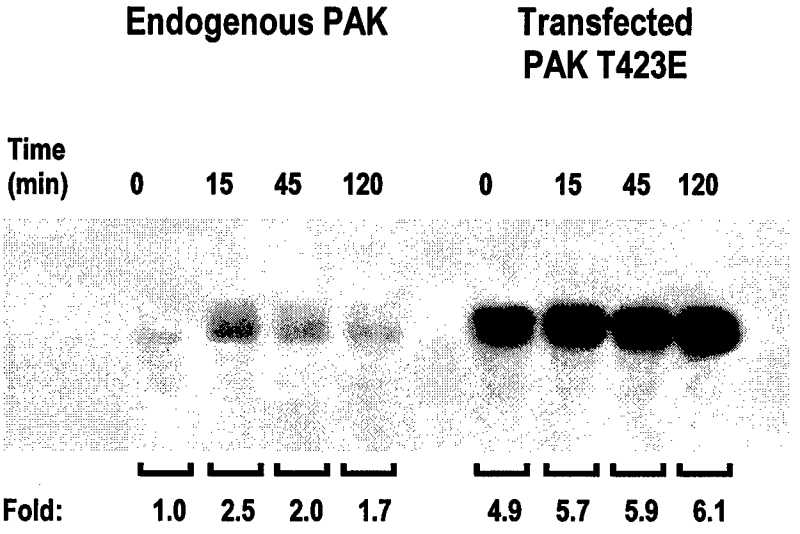

Figure 4: PAK activity during adhesion. BHK-21, control (no virus) and PAK T423E (catalytically active), infected cells where placed in an adhesion assay and lysed at various time points. Cell lysates were run out on a gel containing p47 peptide and an in-gel kinase assay was performed to detect PAK activity (5). Constitutively active PAK shows high activity at all time points. However, control cells show little activity at time 0 with a dramatic increase in PAK activity at 15 min. Note the decrease in PAK activity at the later time points, or when cells are fully spread. This demonstrates that adhesion alone is sufficient to activate PAK and that PAK activity is highest during cell spreading.

Conclusion

Cell adhesion, migration and invasion play a critical role in understanding tumor metastasis. Comprehending cytoskeleton dynamics is pivotal in understanding the complexities of metastasis. Thus, our studies have first focused cell on spreading in order to understand what is occurring at the moving edge of the cell. In this report we provide evidence that the small GTPase Rac1 is important for cell spreading, a process necessary for migration. Rac 1 activity is high early during cell spreading and decreases to base line levels in the later stages of spreading. Both dominant negative and constitutively active Rac1 impair spreading, suggesting the necessity of the GTPase to cycle (turn on, then off) in order for the cell to complete its normal function.

To understand how small GTPases regulate cytoskeleton dynamics, we examined the effect of PAK, an effector molecule of Rac and Cdc42, had on cell spreading. Recently, our lab has shown that PAK localizes to Rac induced membrane ruffles (22) and mutationally active forms can cause cytoskeleton changes (23). This data suggest a role for PAK in cytoskeleton remodeling. Data presented in this report further demonstrate the importance of PAK and suggest a possible mechanizem by which it influences the cytoskeleton. Activated PAK inhibits cell spreading and decreases myosin phosphorylation, consistence with the inability of the cells to spread. We describe a novel target for PAK, MLCK. PAK phosphorylates MLCK and inhibits its activity. Aim 2 in our grant proposal was to determine if PAK effected myosin phosphorylation (Technical Objective 2: task 3 and 4). We believe that these results satisfy the objective of Aim 2.

At present we are working on Aim 3 in our proposal, which is to examine the importance of PAK-myosin interactions on breast cancer cell migration. With the development of the viral gene expression system we are now able to transfect breast cancer cells with high enough transfection efficiency to do migration assays in Boyden chambers (Technical Objective 3: task 5 and 6). These experiments are currently ongoing.

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Inhibition of Myosin Light Chain Kinase By p21-Activated Kinase (PAK) During Cell Spreading

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Cytoskeletal remodeling is crucial in many cellular events, including cell adhesion, spreading and motility [1-3]. The Rho family of small GTPases (Rho, Rac and Cdc42) have been implicated as critical regulators of cytoskeletal changes [4,5]. p21-activated protein kinase 1 (PAK1), an effector molecule activated by Rac and Cdc42, localizes to Rac-induced membrane ruffles [6] and mutationally activated PAKs induce cytoskeletal rearrangement [7,8], suggesting a role for PAK in cytoskeletal dynamics. However, the targets of PAK1 and their specific roles in regulating cytoskeletal structures are not understood. Cytoskeletal dynamics are modulated in large part by actin and myosin II [9-11]. Since actin-myosin II interactions are regulated by myosin light chain phosphorylation (MLC-P), we examined the effects of PAK on MLC-P during cell spreading. Here we show that an activated mutant of PAK1, but not wtPAK1, inhibits cell spreading and MLC-P. In vitro and in vivo studies revealed a novel target for PAK, myosin light chain kinase (MLCK), the enzyme that phosphorylates the 20 kDa light chain of myosin II. Activated PAK phosphorylates MLCK and inhibits its ability to phosphorylate Ser 19 of MLC. An important component of cytoskeletal regulation by PAK thus appears to be its ability to modulate myosin II activity through phosphorylation and downregulation of MLCK.

Cell adhesion and cell spreading on extracellular matrix leads to active cytoskeletal remodeling that is dependent on both actin polymerization and actin-myosin II interactions [2,9,12]. Cell adhesion also results in activation of the Rac- and Cdc42-dependent effector p21-activated kinase (PAK 1) [13]. Therefore, BHK-21 cells expressing wild type PAK1 (wtPAK1) or a catalytically active PAK1 (T423E) were placed on fibronectin-coated cover slips and allowed to adhere and spread for 2 hr. Cells were then fixed and examined using fluorescence microscopy. Non-transfected control cells, LacZ virus controls, and cells expressing wtPAK1 attached and

spread normally over the 2 hr time course (average of 80% cells spread, n=271). Phalloidin staining revealed a typical fibroblast-like morphology, with numerous stress fibers (Fig. 1A). Cells expressing PAK1 (T423E) attached to fibronectin normally, but cell spreading was dramatically reduced (average of 23% cells spread, n=303) and the cells exhibited a non-spread, rounded morphology for the duration of the experiment (Fig. 1A).

Postmitotic cell spreading has been suggested to be an actomyosin-mediated event [2,12]. Actin-myosin II interactions are regulated by MLC-P [14,15] and MLC-P on Ser-19 is increased during postmitotic cell spreading at the moving edge, with a subsequent return to baseline in completely spread cells [12,16]. Consistent with this, we have observed that BHK-21 cells exhibit myosin II staining at the spreading edges (data not shown). In order to confirm the involvement of myosin II in spreading of BHK-21 cells, we treated cells with inhibitors of myosin or MLCK. BDM has been reported to reversibly inhibit nonmuscle myosin ATPase activity and been shown to inhibit postmitotic cell spreading [12]. BDM (20mM) inhibited BHK-21 cell spreading for up to 90 min (Fig. 1B). This inhibition was reversible: when BDM was washed out after 45 min, spreading resumed and occurred normally (Fig. 1B). The inhibition of cell spreading by BDM was dose dependent over the concentration range of 2 to 50 mM (data not shown). Similarly, BHK-21 cell spreading was inhibited with the MLCK inhibitor ML-7 [17] (data not shown).

Since expressing PAK1 (T423E) or inhibiting myosin ATPase activity directly with BDM or indirectly with ML-7 inhibited cell spreading, we analyzed the effect of PAK1 expression on MLC-P. Control BHK-21 cells or cells transfected with PAK1 (T423E) were allowed to attach and spread on a fibronectin matrix and lysed at various times. Immunoblot analysis was then performed using an antibody that specifically recognizes the Ser-19 phosphorylated form of myosin light chain [18]. Nontransfected control cells show a gradual increase in MLC-P, with the

maximum increase at 45 min, during cell spreading (Fig. 2). Cells transfected with control plasmids (Lac Z or wtPAK1) exhibited increases in MLC-P similar to that of the non-transfected cells (data not shown). In contrast, cells expressing PAK1 (T423E) show substantially reduced MLC-P at all time points, consistent with the inability of these cells to spread (Fig. 2). These data suggest that catalytically active PAK1 acts in vivo to inhibit phosphorylation of myosin light chain on Ser-19.

The calcium-calmodulin-dependent MLCK phosphorylates Ser 19 of MLC, and is known to directly regulate the force generating ability of myosin II in vivo [1,19,20]. Previous experiments have shown that MLCK is a substrate for other protein kinases and that phosphorylation can increase [21] or decrease MLCK activity [22-24]. Therefore, we investigated the possibility that PAK1 (T423E) directly phosphorylates and regulates MLCK activity. In vitro phosphorylation experiments demonstrated that PAK1 can directly phosphorylate MLCK (Fig. 3) and that this phosphorylation is independent of calmodulin binding to MLCK (data not shown). Furthermore, the catalytic activity of MLCK phosphorylated by PAK1 is decreased by ~50 % when assayed at a saturating calmodulin concentration (Fig. 3).

We next examined the ability of PAK to inactivate MLCK in vivo by transfecting Hela cells with wild type or PAK1 (T423E). Western blot analysis confirmed equal expression of wild type or PAK1 (T423E). The cells were lysed and endogenous MLCK was immunoprecipitated and assayed for activity. MLCK immunoprecipitated from cells expressing PAK1 (T423E) showed substantially decreased activity when compared to MLCK immunoprecipitated from control- or wtPAK1-expressing cells (Fig.4). These data support the in vitro data indicating that PAK-mediated phosphorylation of MLCK inhibits its activity, thereby resulting in a decrease of MLC phosphorylation.

The dynamic nature of cell rearrangement and motility requires complex yet coordinated regulation by Rho, Rac, and Cdc42 GTPases [3,4,25]. Recently, the Rho effector molecule Rho kinase has been shown to phosphorylate myosin phosphatase and inhibit its activity [26], and may directly phosphorylate the MLC as well [27]. Both activities serve to increase MLC-P and stimulate contractility [26,27]. This regulatory activity of Rho may be important in the contractile events necessary for cell spreading [4,25-27] and maintenance of the rigidity characteristic of fully spread, stationary fibroblasts. In contrast to Rho, Rac and Cdc42 appear to regulate actin rearrangements that are important in the early stages of cell spreading, as well as dynamic morphological changes that are associated with cell migration [14]. Our results identify a specific target for PAK, MLCK, which is a known regulator of myosin II function. They also describe a previously unknown mechanism for regulating MLCK activity and the intracellular level of MLC-P. Moreover, the regulation of MLCK activity and MLC-P appears to be an important component of Rac- and Cdc42-dependent cytoskeletal remodeling in spreading cells. Since Rho kinase and PAK have opposing effects in MLC-P, the integrated cellular response to Rho versus Rac/CDC42 activation may depend on the intracellular location and extent of myosin light chain phosphorylation.

Methods

Transfection of cells with Semliki Forest Virus: The cDNA encoding wtPAK1 or a constitutively active form of PAK1 in which threonine 423 is mutated into a glutamic acid residue (T423E) were expressed in cells using the Semliki Forest Virus (SFV) Gene Expression System (Life Technologies, Gaitherburg, MD). Briefly, the cDNAs were PCR amplified using primers that contained a BamH1 restriction enzyme site and a myc tag at the 5' end. These constructs were subcloned into the BamH1 site of the Semliki Forest vector pSFV3. In vitro transcription of linearized pSFV3 constructs and pSFV-Helper2 was performed using SP6 RNA polymerase. RNA transfection of BHK-21 cells was done by electroporation as previously described [28], yielding recombinant viral stocks of approximately 10^7 plaque-forming units (pfu)/ml. Viral stocks were stored at -80C until use. Virus was activated per manufacturer's instruction, and BHK-21 and Hela cells were infected in serum-free media. Transfection efficiency of recombinant virus was routinely >95% in BHK-21 cells and >50% in Hela cells. Cells were allowed to express protein for 6 to 8 hour after infection in serum-free media before use in experiments.

Cell Adhesion Assay: Cell adhesion assays were performed as previously described [29]. In brief, cells were suspended in basal media (GMEM, Life Technologies) containing no serum and seeded in 6-well plastic microtiter plates containing cover slips pre-coated with 20 μg/ml fibronectin. Cells were allowed to adhere and at various time points were fixed in 4% paraformaldehyde prior to immunofluorescence analysis. For inhibition studies, various concentrations of BDM (2,3-butanedione monoxime, Sigma) and ML-7 (Calbiochem) were added to the media.

Immunofluorescence: Cells attached to coverslips were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.5% Triton X-100 for 20 min, then incubated with anti myc (9E10) at 1:300

or anti-Gal at 1:5,000 (Promega, Madison, WI) for 1 hr. Cells were then incubated for 1 hr with rhodamine phalloidin 1:500 (Sigma Chemical Co.) and/or FITC-conjugated anti-mouse IgG 1:300 (Cappel Laboratories, Cochranville, PA), washed with PBS, and mounted in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Slides were examined using a Nikon Labphot-ZDFX-DX epifluorescence microscope, and images photographed with a 35-mm camera back and Kodak Tmax film.

Immunoblots: Cell lysate (30µl) from control (nontransfected) or transfected BHK-21 cells (35 mm dish) were run on 15% SDS-PAGE gels, transferred to PVDF membrane using a semi-dry transfer apparatus, stained with a 2% Ponseau S solution to check transfer, then rinsed in water. After blocking, blots were incubated with anti-Ser 19 phosphorylated myosin light chain antibody pp2b for 1 hr [18]. Protein bands were visualized with horseradish-peroxidase-conjugated anti-rabbit IgG (Pierce) and chemiluminescence (Pierce).

Phosphorylation assays: Purified smooth muscle MLCK (5.2µg) was incubated at 30°C for 1 hr by itself (autophosphorylation control) or with 0.5 µg of constitutively active recombinant GST-PAK1 (prepared as in [30]) in buffer containing 10 mM $MgCl_2$, 2 mM DTT, 0.1 mM ^{32}P-labeled ATP (specific activity ~2000 cpm/pmol), and 20 mM Tris-HCl, pH 7.5. Aliquots were removed at various times and analyzed by SDS PAGE and autoradiography. Samples were also tested for MLCK activity by incubating 50 ng of autophosphorylated- or PAK1-phosphorylated MLCK at room temperature as above, with the further inclusion of 0.5 mM $CaCl_2$, 10-7M calmodulin, 5 mM DTT and 10 µg of purified myosin light chains. Aliquots were removed at various times and subjected to SDS PAGE. The bands representing the myosin light chains were then excised and counted. To quantify changes in MLCK activity in cells, MLCK was immunoprecipitated from non transfected HeLa cells or HeLa cells expressing Lac Z, wtPAK1 or PAK1 (T423E) as

previously described [23] using affinity purified goat antibodies to MLCK[20] and Protein A-Sepharose. The beads were washed extensively and then assayed for MLCK activity as described above.

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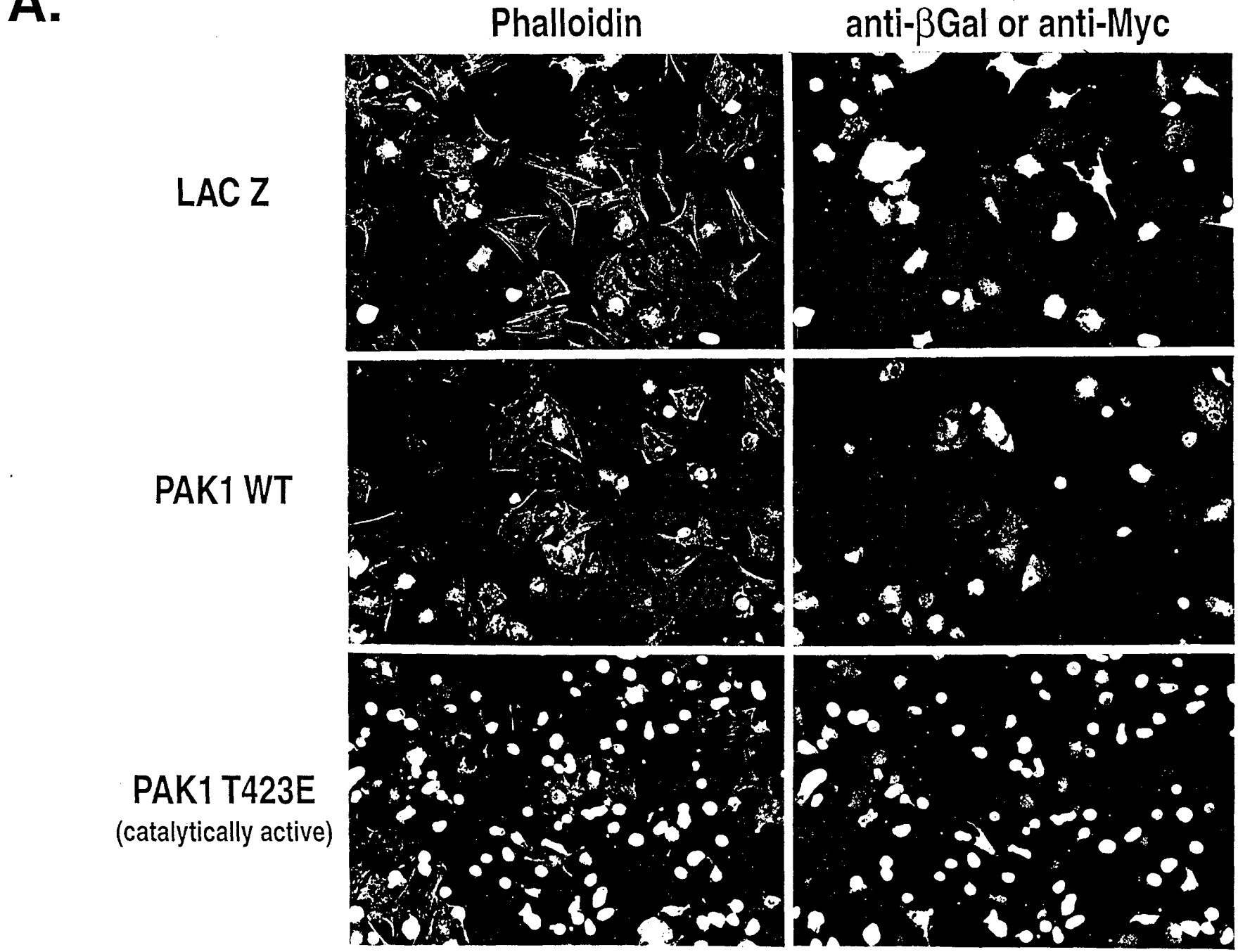
A.
Phalloidin
anti-βGal or anti-Myc
LAC Z
PAK1 WT
PAK1 T423E
(catalytically active)

Figure 1A

B.

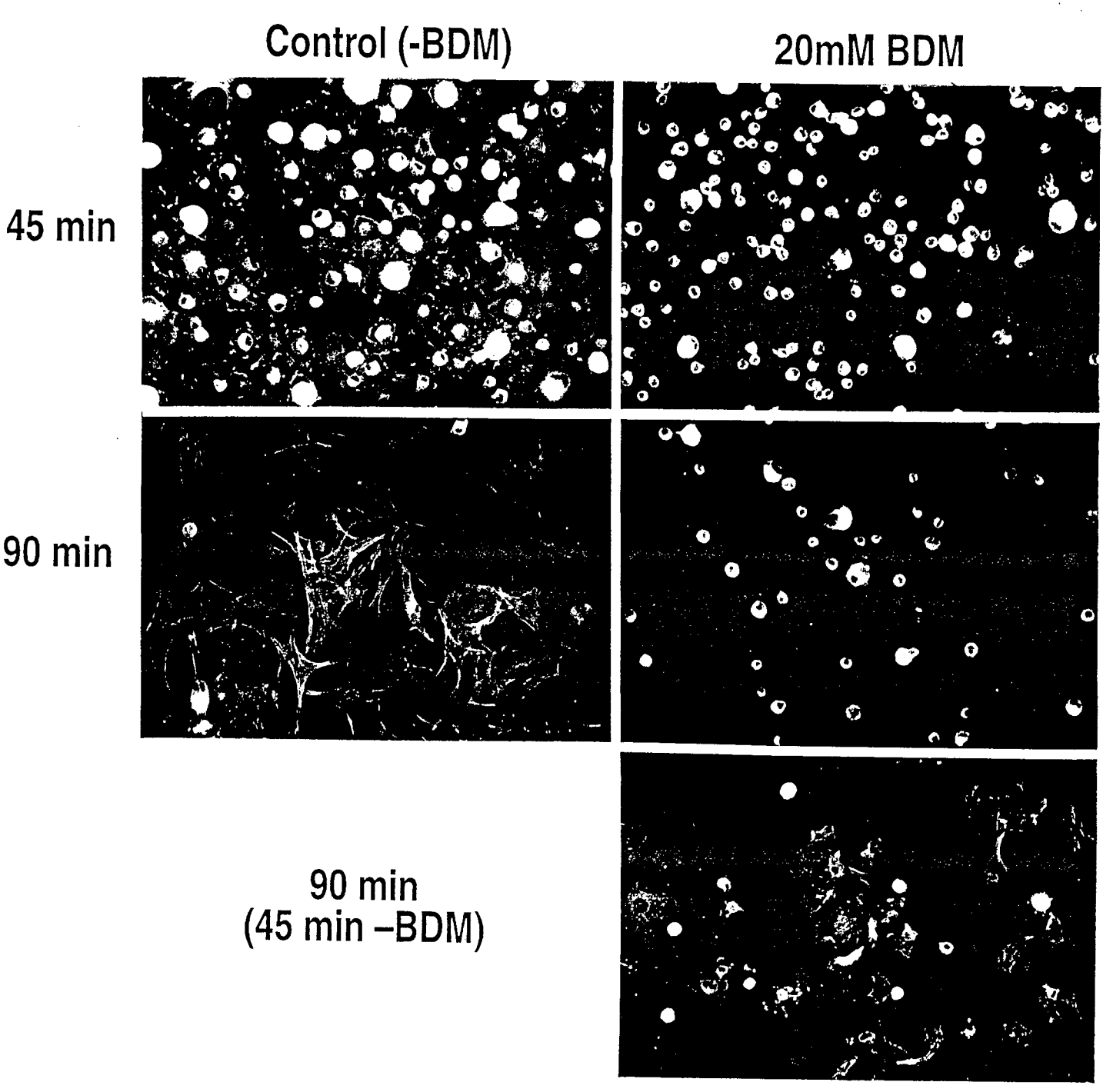

Figure 1B

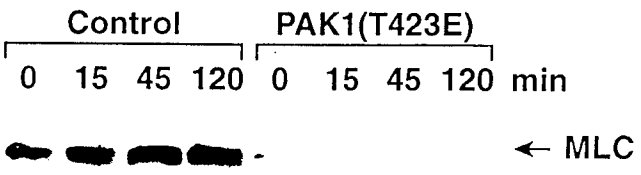

Figure 2

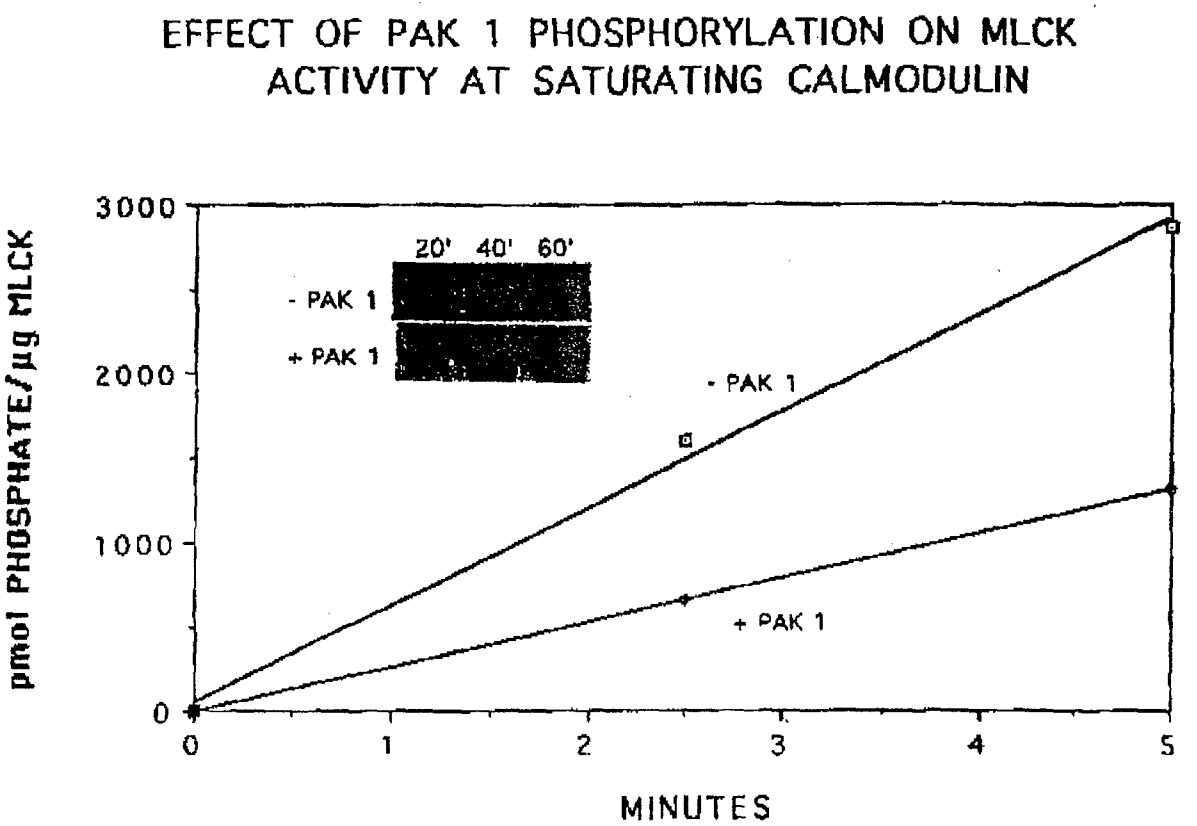
EFFECT OF PAK 1 PHOSPHORYLATION ON MLCK ACTIVITY AT SATURATING CALMODULIN
pmol PHOSPHATE/µg MLCK
3000
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Figure 3

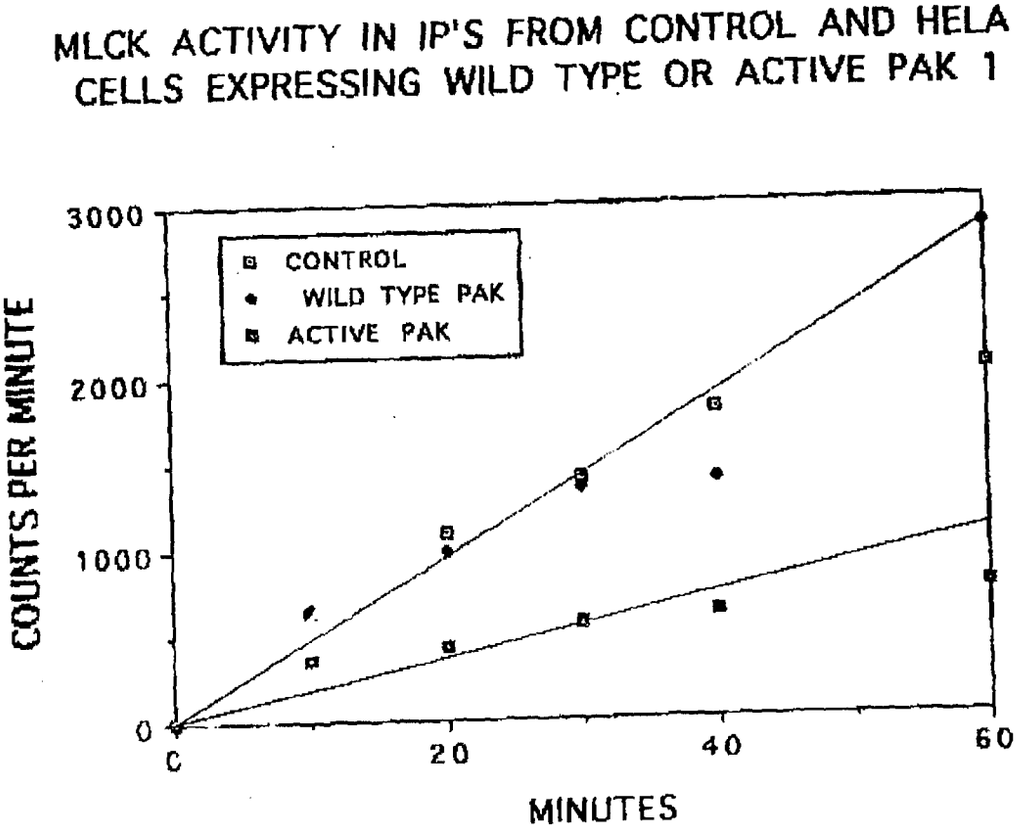
MLCK ACTIVITY IN IP'S FROM CONTROL AND HELA CELLS EXPRESSING WILD TYPE OR ACTIVE PAK 1
CONTROL
WILD TYPE PAK
ACTIVE PAK
COUNTS PER MINUTE
3000
2000
1000
0
0
20
40
60
MINUTES

Figure 4

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